Characterization of the Collagen–Vinyl Graft Copolymers Prepared by the Ceric Ion Method. II. Infrared Spectra and Electron Microscopy

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Synopsis

Collagen powder and goat skins were grafted with different vinyl monomers using the ceric ion technique. The graft copolymers were characterized by infrared spectra and electron microscopy. The collagen–vinyl graft copolymers were hydrolyzed by both acid and enzymatic hydrolysis, and the grafted vinyl polymer side chains were isolated. In the grafted poly(methyl methacrylate) (PMMA) side chains isolated by acid hydrolysis, the characteristic amide absorption bands at 1550 and 1660 cm^{-1} were not seen prominently. However, in PMMA side chains isolated by enzymatic methods, the amide absorption bands were more prominent as these isolated side chain polymers contained longer fragments of the peptide backbone attached to them. Electron-microscopic observations of grafted collagen fibrils and ultrathin sections of grafted goat skin fibrils did not show any cross-striations. These various evidences indicate that the polymers formed on collagen have penetrated into the fibrils and that they were chemically bound to the collagen molecules.

INTRODUCTION

The grafting of vinyl polymers through ceric ion initiation on collagen was reported in the previous paper[1] in which proof for real chemical grafting was established by different methods of characterization of graft copolymers. The grafted polyvinyl side chains were separated by selective hydrolysis of the collagen backbone with hydrochloric acid or proteases in order to characterize the graft copolymers. The present paper reports the infrared spectra of the isolated grafts and electron-microscopic observations of the grafted collagen fibrils and ultrathin sections of fibrils.

Infrared spectroscopy has been found to be a valuable tool in studying graft copolymerization reactions. This technique has been extensively used[2–6] to establish proof of grafting, to ensure complete removal of nongrafted products, and to determine the structure of the grafted products.

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Knowledge of the location of the grafted polymer is also highly important both for elucidation of the mechanisms of the grafting process and to correlate with the properties of the grafted fibers. Although studies on the location of the grafted polymer in fibers are somewhat scarce, excellent use was made of the electron microscope in this field by Arthur, Rollins, and their co-workers[6–11] and Liggett et al.[12] with grafting onto cotton and by Kaeppner and Huang[13] with rayon. Andrews and his co-workers[14,15] and Ingram et al.[16] have used the electron microscope for location of polyacrylonitrile (PAN) and polystyrene in wool, respectively. The electron microscope has been widely used in studies of connective tissue, and it has contributed much to the knowledge of the structure and distribution of collagen fibrils in skin. With the electron microscope it is possible to determine the location of grafted polymer in the collagen fiber in a much more detailed manner. Electron-microscopic observations of grafted collagen fibrils and ultrathin sections of fibrils were, therefore, undertaken in the present investigation with the aim of locating the graft copolymer in the collagen fibrils.

EXPERIMENTAL

Materials

Monomers. Methyl methacrylate (MMA), methyl acrylate (MA), and *n*-butyl acrylate (BA) from Rohm and Haas, U.S.A., and acrylonitrile (AN) from BDH, were purified by standard methods as described in the previous paper.[1]

Enzymes. Pronase, B grade (Cal-Biochem, U.S.A.), trypsin (A. Costantino & C., Favria, Italy), and pepsin (A. Costantino & C., Favria, Italy) were used without further purification.

Chemicals. Ceric ammonium nitrate (CAN) and ceric ammonium sulfate (CAS) (Puriss, Fluka) were used without further purification. Other chemicals used were reagent grade.

Preparation of Collagen–Vinyl Graft Copolymers

In the case of collagen powder, the graft copolymers were prepared as described in our previous papers.[17–19] For grafting on skins, an emulsion

TABLE I
Characterization of Graft Copolymers Studied

Substrate grafted	Monomer	Sample Code	Per cent grafting, %
Hide powder	MMA	GC_1	159.6
Hide powder	MMA	G_1C_1	20.0
Hide powder	MMA	G_2C_1	120.0
Goat skin	MMA	GS_1	107.0
Goat skin	MA	GS_2	45.0
Goat skin	*n*-BA	GS_3	64.0
Goat skin	AN	GS_4	44.0

polymerization technique[20] was followed. The graft copolymers were thoroughly extracted with the appropriate solvents to remove the occluded and loosely bound homopolymers.

Content of Collagen in the Graft Copolymers

The collagen content of the graft copolymers was calculated from the content of total nitrogen (by multiplying the nitrogen content by 5.6). In the case of AN-grafted samples, the percentage of collagen was calculated from the arginine value (by multiplying the arginine content by 11.6).

Per Cent Grafting

The per cent grafting was calculated as follows:

$$\frac{\text{otal weight of graft copolymer} - \text{weight of collagen}}{\text{weight of collagen}} \times 100.$$

The per cent grafting in the various products used in these studies is given in Table I.

Isolation of grafts

By Acid Hydrolysis. Acid hydrolysis was carried out by heating the graft copolymer with 6*N* HCl for 18 hr. The insoluble graft was filtered off, washed, and dried.

By Enzymatic Hydrolysis. (a) Digestion with pronase. The grafted vinyl polymer chains of the collagen–graft copolymers were isolated by pronase digestion as described in the previous paper.[1]

(b) Digestion with pepsin: Samples of collagen–poly(MMA) graft copolymers (0.1–0.3 g) after heat denaturation in boiling water were digested with pepsin in 100 ml water adjusted to pH 2 by dilute hydrochloric acid. The ratio of collagen to enzyme was 10:1. After digestion, the insoluble residues were removed by centrifugation, washed with warm water, and dried.

(c) Digestion with trypsin: Samples of collagen–poly(MMA) graft copolymers (0.1–0.3 g) after heat denaturation in boiling water were adjusted to pH 8 by dilute sodium hydroxide and digested with trypsin. The ratio of collagen to enyme was 10:1. After digestion, the insoluble residue was removed by centrifugation, washed with warm water, and dried.

Infrared Spectra

The infrared spectra of the grafts isolated by acid and enzymatic hydrolysis and the corresponding homopolymers were measured with a Perkin-Elmer Model 337 grating infrared spectrophotometer in the form of potassium bromide (KBr) pellets (500 mg) containing 2–6 mg powdered polymers.[21]

Electron Microscopy

Dispersion Technique. Samples of collagen powder grafted with poly(MMA) were used for this purpose. A small quantity of the collagen graft

copolymer was disintegrated in a glass fiber disintegrator using deionized water at 4°C, and a drop of the suspension of the fibril was placed on collodion-covered specimen grids. After drying over calcium chloride in a desiccator, the specimen was shadowed with gold–palladium (60:40) at an angle of 14° and examined in a Siemens Elmiskop I.

Ultrathin Sectioning. Goat skins grafted with poly(MMA), poly(MA), poly(BA), and poly(AN) using the ceric ion aqueous emulsion technique were used for this purpose. The dried grafted goat skin samples were cut into rectangular pieces approximately 5 mm × 2 mm in size. The universally employed methacrylate method was used for embedding the grafted fiber bundles. After suitable cleansing and dehydration procedures, the block was trimmed to have a perfectly plane square face with side equal to 0.5 mm. Sections about 250 Å thick were cut, using a Porter-Blum ultramicrotome fitted with a glass knife. Then the sections were floated in 40% acetone and spread with xylene vapors.

The sections were mounted on the carbon-coated grids. The samples mounted on the specimen grids were stained with a 1% solution of phosphotungstic acid in a 1:1 ethyl alcohol–water mixture. All the samples were scanned in a Siemens Elmiskop I operated at 80 kV and using both the condensers and an objective aperture of 50 microns. Typical fields were photographed at instrumental magnifications of 6000× and 8700×; the calibration of the instrument was checked by using polystyrene latex particles supplied by the Dow Chemical Co., Midland, Mich.

RESULTS AND DISCUSSION

Infrared Spectra of Isolated Grafts

The infrared spectra of the polyvinyl side chains isolated from collagen graft copolymers by acid and enzymatic hydrolysis were expected to give additional evidence as to whether actual grafting had taken place. When the products are true graft copolymers, the infrared spectra of the isolated polyvinyl side chains will show typical bands of amino acid residues and those of the grafted polymer. The infrared spectra of a number of grafted side chains isolated from collagen graft copolymers by both acid and enzymatic hydrolysis were therefore examined. Since the extent of hydrolysis of denatured collagen will also depend on the nature of the enzyme, a number of proteolytic enzymes were used in the present study to digest the collagen backbone of the graft copolymers.

As a representative sample, the infrared spectra of the isolated poly(MMA) side chains obtained by enzymatic and acid hydrolysis along with the methyl methacrylate homopolymer are shown in Figures 1–5. The infrared spectrum of untreated collagen is given in Figure 6. In the grafted side chains isolated by acid hydrolysis, the characteristic amide absorption bands at 1550 and 1660 cm^{-1} are not seen prominently, whereas in the grafts isolated by the enzymatic methods, the characteristic absorption bands of the amide groups are very prominent. This is to be expected

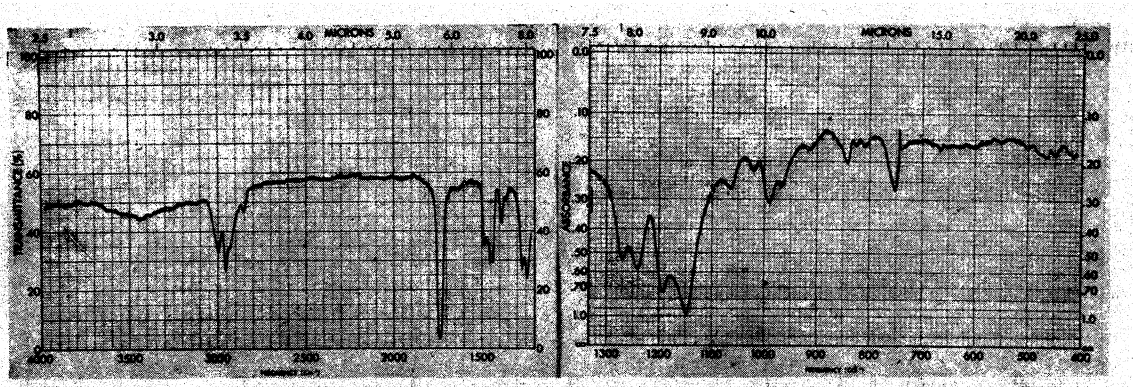

Fig. 1. Infrared spectrum of PMMA homopolymer prepared by the ceric ion method.

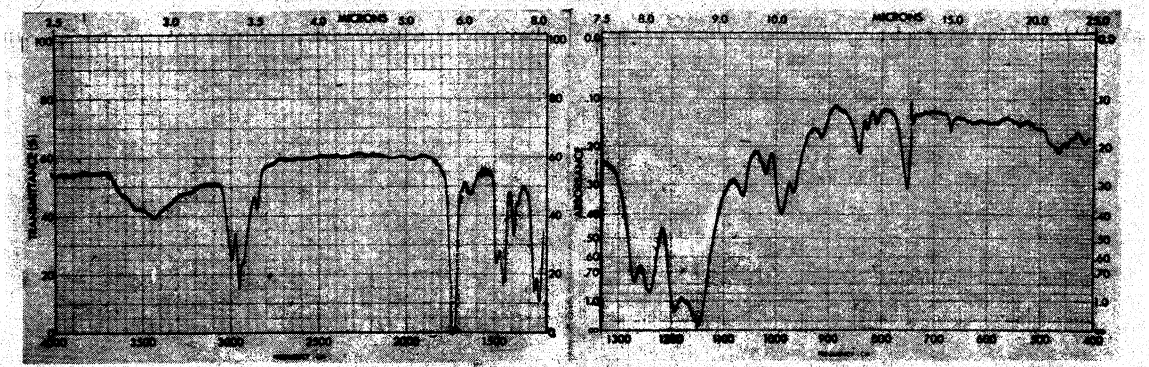

Fig. 2. Infrared spectrum of PMMA grafts isolated from collagen–PMMA graft copolymer (sample GC_1) by acid hydrolysis.

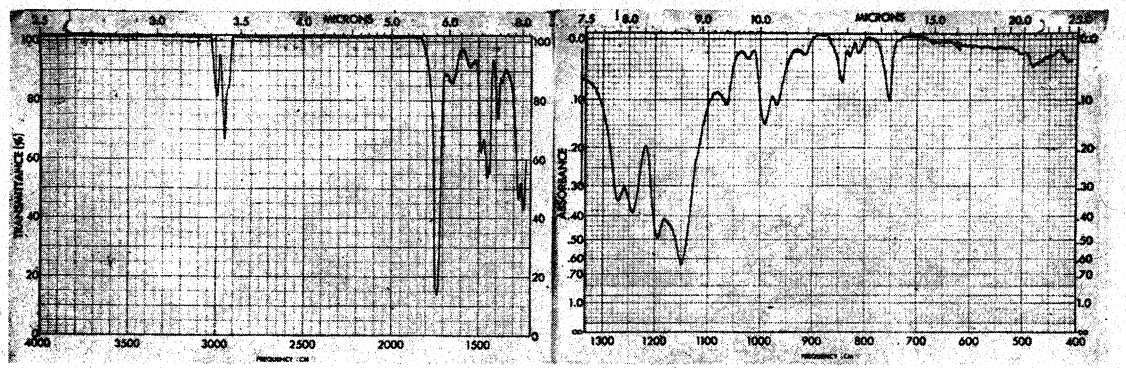

Fig. 3. Infrared spectrum of PMMA grafts isolated from collagen–PMMA graft copolymer (sample GC_1) by pronase digestion

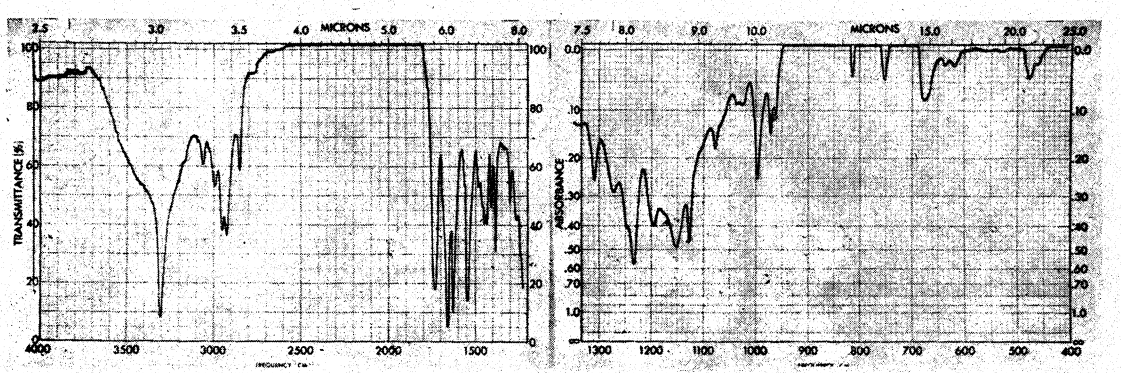

Fig. 4. Infrared spectrum of PMMA grafts isolated from collagen–PMMA graft copolymer (sample GC_1) by pepsin digestion.

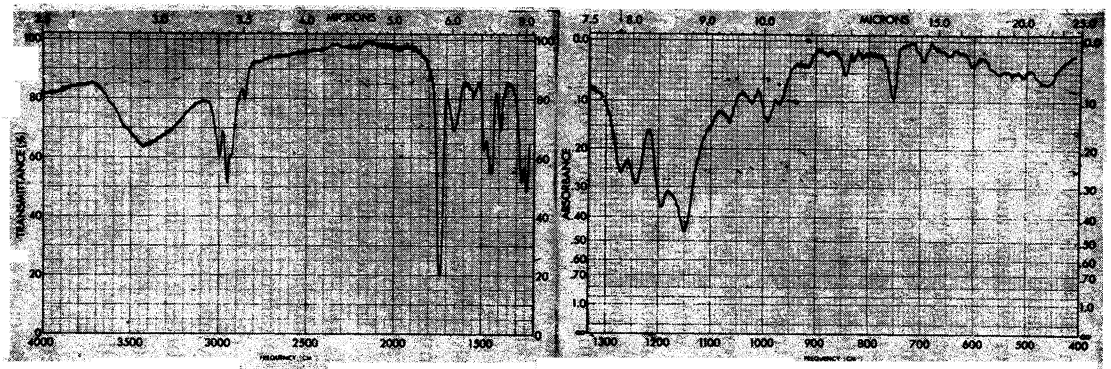

Fig. 5. Infrared spectrum of PMMA grafts isolated from collagen–PMMA graft copolymer (sample GC_1) by trypsin digestion.

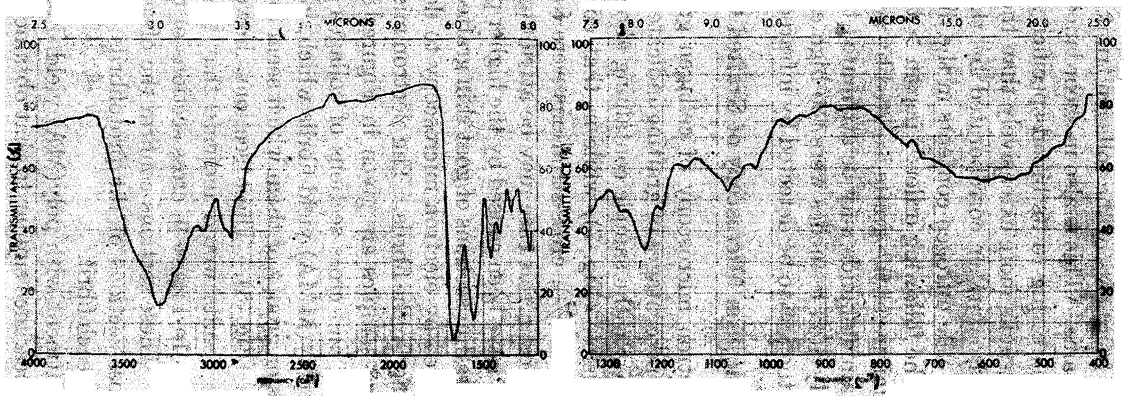

Fig. 6. Infrared spectrum of untreated collagen film.

because the proteolytic enzymes will give rise to longer fragments attached to the end of the grafted polymer, and hence the absorption bands for the peptide amide bands will be more prominent in these cases. The absorption bands of the amide groups are more prominent in the case of grafts isolated by pepsin and trypsin digestion than in the case of grafts isolated by pronase. This shows that the grafts isolated by pepsin and trypsin have longer fragments of the collagen trunk attached to the end of grafted polymer chains. This is also in accordance with the specificity of the enzymes; pronase, owing to its broad specificity, is capable of hydrolyzing the collagen trunks more extensively than the other two enzymes. These results were also further corroborated by the nitrogen contents of the isolated grafts; the nitrogen contents in the different isolated grafts were found to be in the following order: pepsin > trypsin > pronase > acid hydrolysis. The failure of the grafts isolated by acid hydrolysis to show the characteristic amide bands may be attributed to the fact that the molecular weight of polyvinyl branches were too high and the attached amino acid residues were too few to be detected by infrared spectroscopy.

Electron Microscopy of Grafted Collagen Fibers and Goat Skins

Electron microscopy of collagen grafted with poly(MMA) at two different percentages of grafting indicated variations in the appearance of the fibrils. In the sample containing about 20% polymer, cross-striations are still visible (Fig. 7), while in that containing a high amount of polymer (above 100%), cross-striations were found to be absent. The absence of striations in this case may be attributed to the coating of the fibrils which might have been caused by the higher per cent grafting.

Ultrathin sections of goat skin grafted with different vinyl polymers were examined by electron microscopy to determine the location of the grafted polymer in the fibrils. The electron micrographs of ultrathin sections of the grafted samples are given in Figures 8–11. In these figures, both longitudinal and cross sections of collagen fibrils are shown. Samples treated with poly(MMA) yield fibrils which, in longitudinal section, exhibit protofibrils or groups of them which seem to be spiralling around the axis of the fibrils (Fig. 8). These sections, however, do not show cross-striations. These results would indicate that the polymer has penetrated into the fibrils. In Figure 9, cross sections of the fibrils from the same sample are seen. These sections confirm the observation on the longitudinal section, as cross sections of the protofibrils or groups of protofibrils can be seen within each fibril.

Treatment with poly(MA) yielded sections of collagen fibrils which had an appearance similar to that obtained in the case of treatment with poly(MMA).

Figures 10 and 11 are electron micrographs of sections of collagen fibrils from samples treated with poly(BA). In Figure 10, the longitudinal sections of the fibrils appear completely coated. Figure 11, which shows cross sections of fibrils, confirms this observation. One can conclude from this that polymer deposition has taken place throughout the fibrils. Sections of

Fig. 7. Electron micrograph of collagen fibrils from hide powder grafted with 20% PMMA (sample G_1C_1) shadowed with palladium-gold. 22,500×

Fig. 8. Electron micrograph of longitudinal section of fibrils from goat skin grafted with 107% PMMA (sample GS_1) stained with 1% solution of phosphotungstic acid in 50% alcohol (PTA). 16,300×

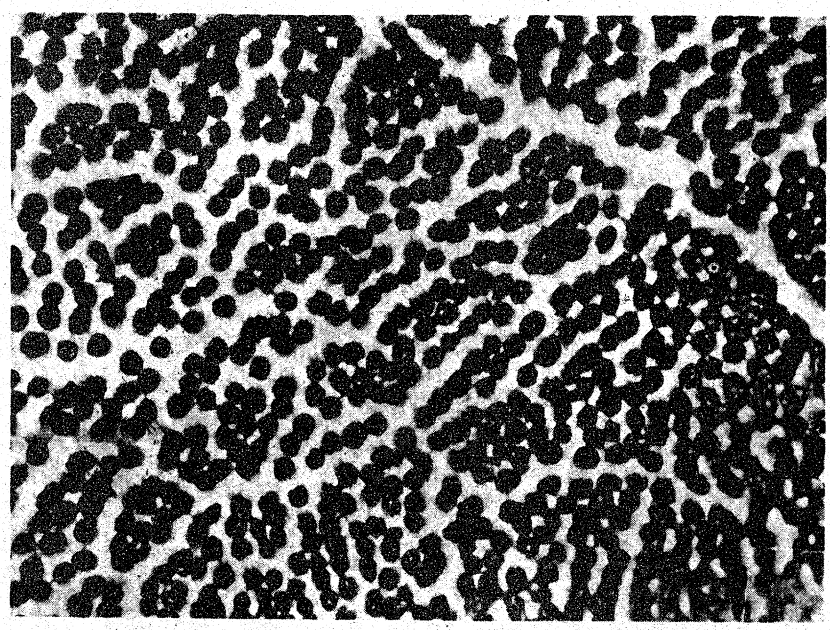

Fig. 9. Electron micrograph of cross section of fibrils from goat skin grafted with 107% PMMA (sample GS_1) stained with 1% PTA. 16,300×

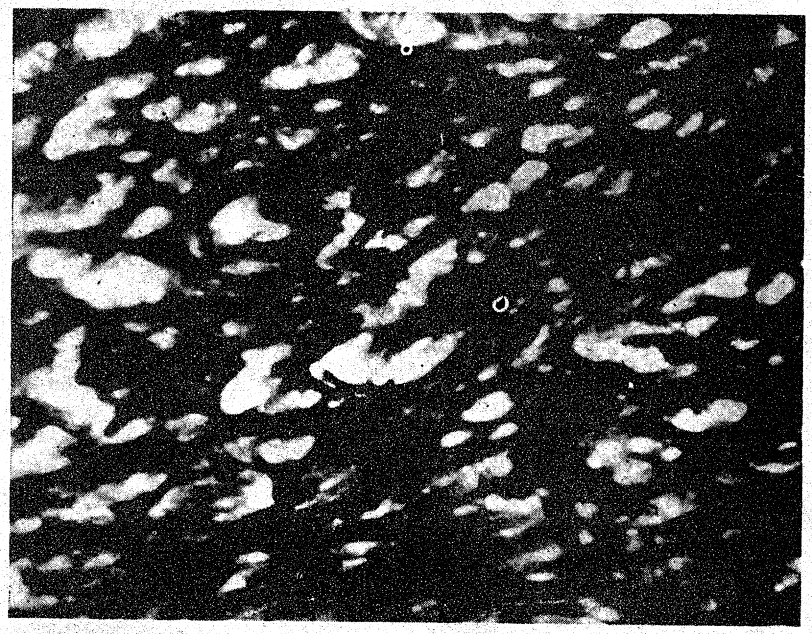

Fig. 10. Electron micrograph of longitudinal section of fibrils from goat skin grafted with 64% PBA (sample GS_3) stained with 1% PTA. 17,400×

collagen fibrils grafted with poly(AN) were found to be similar in appearance of those obtained from poly(BA) grafted samples.

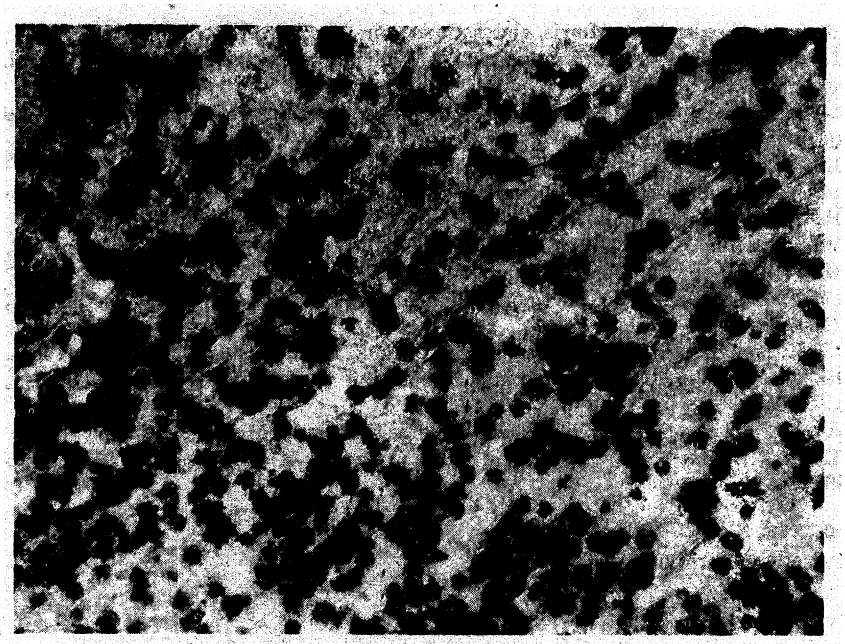

Fig. 11. Electron micrograph of cross section of fibrils from goat skin grafted with 64% PBA (sample GS_3) stained with 1% PTA. 16,300 ×.

Hence it would appear that the polymers studied in this investigation fall into two groups, each of them giving a different appearance of the fibrils in the electron microscope. The combined physical and chemical evidence indicates that the polymers formed on collagen are chemically bound to the collagen molecules and that these polymers have penetrated into the fibrils resulting in the masking of the cross-striations.

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References

1. K. Panduranga Rao, K. Thomas Joseph, and Y. Nayudamma, *J. Polym. Sci. A-1*, **9**, 3199 (1971).
2. J. C. Arthur, Jr., and R. J. Demint, *Text. Res. J.*, **31**, 988 (1961).
3. S. Das Gupta, *Can. Spectrosc.*, **12**, 1 (1967).
4. K. Arai, M. Negishi, and T. Okabe, *J. Appl. Polym. Sci.*, **12**, 2585 (1968).
5. S. P. Potnis, S. M. Shetty, and J. Prakash, *Text. Res. J.*, **40**, 389 (1970).
6. J. C. Arthur, Jr., and R. J. Demint, *Text. Res. J.*, **30**, 505 (1960).
7. R. J. Demint, J. C. Arthur, Jr., A. R. Markezich, and W. F. McSherry, *Text. Res. J.*, **32**, 918(1962).
8. F. A. Blouin and J. C. Arthur, Jr., *Text. Res. J.*, **33**, 727 (1963).
9. M. L. Rollins, A. T. Moore, W. R. Goynes, J. H. Carra, and I. V. deGruy, *Am. Dyestuff Rptr.*, **54**, 512 (1965).

10. F. A. Blouin, N. J. Morris, and J. C. Arthur, Jr., *Text. Res. J.*, **36**, 309 (1966).
11. M. L. Rolline, A. M. Cannizzaro, F. A. Blouin, and J. C. Arthur, Jr., *J. Appl. Polym. Sci.*, **12**, 71 (1968).
12. R. W. Liggett, H. L. Hoffman, A. C. Tanquary, and C. Hamalainen, *Amer. Dyestuff Rptr.*, **58**, 25 (1969).
13. W. M. Kaeppner and R. Y. M. Huang, *Text. Res. J.*, **35**, 504 (1965).
14. M. W. Andrews, *J. Roy. Microsc. Soc.*, **84**, 439 (1965).
15. M. W. Andrews, R. L. D'Arcy, and I. C. Watt, *J. Polym. Sci. B*, **3**, 441 (1965).
16. P Ingram, J. L. Williams, V. Stannett, and M. W. Andrews, *J. Polym. Sci. A-1*, **6**, 1895 (1968).
17. K. Panduranga Rao, K. Thomas Joseph, and Y. Nayudamma, *Leath. Sci.*, **14**, 73 (1967).
18. K. Panduranga Rao, K. Thomas Joseph, and Y. Nayudamma, *Leder*, **19**, 77 (1968).
19. K. Panduranga Rao, K. Thomas Joseph, and Y. Nayudamma, *Leath. Sci.*, **16**, 401 (1969).
20. K. Panduranga Rao, Ph.D. Thesis, Madras University, Madras, 1970.
21. E. R. McCall, S. H. Miles, and R. T. O'Connor, *Amer. Dyestuff Rptr.*, **56**, 13 (1967).